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Phenotypic Switching in *Pseudomonas brassicacearum* Involves GacS- and GacA-Dependent Rsm Small RNAs

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The plant-beneficial bacterium *Pseudomonas brassicacearum* forms phenotypic variants *in vitro* as well as *in planta* during root colonization under natural conditions. Transcriptome analysis of typical phenotypic variants using microarrays containing coding as well as noncoding DNA fragments showed differential expression of several genes relevant to secondary metabolism and of the small RNA (sRNA) genes *rsmX*, *rsmY*, and *rsmZ*. Naturally occurring mutations in the *gacS-gacA* system accounted for phenotypic switching, which was characterized by downregulation of antifungal secondary metabolites (2,4-diacetylphloroglucinol and cyanide), indoleacetate, exoenzymes (lipase and protease), and three different *N*-acyl-homoserine lactone molecules. Moreover, in addition to abrogating these biocontrol traits, *gacS* and *gacA* mutations resulted in reduced expression of the type VI secretion machinery, alginate biosynthesis, and biofilm formation. In a *gacA* mutant, the expression of *rsmX* was completely abolished, unlike that of *rsmY* and *rsmZ*. Overexpression of any of the three sRNAs in the *gacA* mutant overruled the pleiotropic changes and restored the wild-type phenotypes, suggesting functional redundancy of these sRNAs. In conclusion, our data show that phenotypic switching in *P. brassicacearum* results from mutations in the *gacS-gacA* system.

Microorganisms sense and respond to environmental changes by using sensor-effector regulatory circuits that modulate gene expression in response to external stimuli. In addition, microorganisms have evolved different strategies to respond and adapt to their environments, such as phenotypic switching, which is used by several bacterial species to generate population diversity, to increase bacterial fitness, and to adapt to fluctuating environments (25, 38). It is well established that some bacteria can generate population heterogeneity from an overall genetically homogeneous population under natural conditions, facilitating the survival of part of the bacterial population in response to environmental fluctuations. Such adaptive phenomena, which enable bacteria to exploit and explore their ecological niches more effectively, have been described for some *Pseudomonas* strains inhabiting the rhizosphere (2, 39). In these bacteria, spontaneous mutations in the *gacS-gacA* two-component system account for the phenotypic variation observed (39).

The GacS-GacA two-component system activates the transcription of one or several genes specifying noncoding small RNAs (sRNAs), which regulate carbon storage and secondary metabolism in gammaproteobacteria (23). The carbon storage regulator (CsrA) and the homologous regulator of secondary metabolism (RsmA) control a wide range of genes by binding, as dimers, to GGA motifs in the 5′ untranslated region of target mRNAs, thereby preventing translation of the mRNAs. Csr/Rsm sRNAs antagonize the activities of their cognate CsrA/RsmA proteins by exposing several GGA recognition motifs, resulting in sequestration of the RNA-binding proteins (22). In *Pseudomonas aeruginosa*, GacA activates specifically and exclusively the expression of the sRNA genes *rsmY* and *rsmZ* (8), whereas in *Pseudomonas fluorescens* CHA0, GacA activates the expression of four noncoding sRNA genes, *rsmX*, *rsmY*, *rsmZ* (all directly), and *rgsA* (indirectly) (14, 20). Deletion of the *rsmY* and *rsmZ* genes in *P. aeruginosa* (21) and of *rsmX*, *rsmY*, and *rsmZ* in *P. fluorescens* CHA0 (20) results in phenotypes that are similar to those of *gacS* and *gacA* mutants. Similar observations have also been reported for the homologous genes in other gammaproteobacteria, such as *Escherichia coli* (42), *Salmonella enterica* (13), *Erwinia carotovora* (24), and *Legionella pneumophila* (34).

Pseudomonas brassicacearum has been described as the major root-associated bacterium in the rhizosphere of *Arabidopsis thaliana* and *Brassica napus* (1, 29). This bacterium frequently undergoes phenotypic switching *in vitro* (12) and in the rhizosphere of different plants grown *in vitro* (2). *In vitro*, the variant form (also termed phase II) appears at the edge of wild-type colonies growing on rich medium. Unlike the wild type (also termed phase I), the variants form flat, nonmucoid colonies and no longer secrete protease and lipase.

Here we identified the regulatory components controlling phenotypic switching in *P. brassicacearum* strain NFM421. Frequent point mutations in the *gacS* or *gacA* gene were found to account for phenotypic variation of this strain and to cause pleiotropic phenotypes that are relevant for biofilm formation and bacterial

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TABLE 1 Strains and plasmids used in this study

Strain(s) or plasmid	Description	Reference or source
Strains		
Escherichia coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL*(Str^r) *endA1* λ$^-$	Invitrogen
GM2163	F$^-$ *dam-13*::Tn*9 dcm-6 hsdR2 leuB6 his-4 thi-1 ara-14 lacY1 galK2 galT22 xyl-5 mtl-1 rpsL136 tonA31 tsx-78 supE44* McrA$^-$ McrB$^-$	
S17-1	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu, Kn::Tn*7*	
Pseudomonas brassicacearum		
Phase I NFM421	Wild type	1
Variants (V1, V2, V3, and V4)	Mutation in *gacA* or *gacS* gene	This study
NFM421 Δ*gacA*	Δ*gacA*	This study
NFM421 Δ*lapD*	Δ*lapD*	This study
Plasmids		
Transcriptional *lacZ* fusions		
pME6016	pVS1-p15A shuttle vector for transcriptional *lacZ* fusions; Tc^r	35
pME6016-rsmX-lacZ	pME6016 derivate containing a transcriptional *rsmX′-′lacZ* fusion; Tc^r	This study
pME6016-rsmY-lacZ	pME6016 derivate containing a transcriptional *rsmY′-′lacZ* fusion; Tc^r	This study
pME6016-rsmZ-lacZ	pME6016 derivate containing a transcriptional *rsmZ′-′lacZ* fusion; Tc^r	This study
Overexpressions		
pME6032	NruI-EcoRI *lacI*^{q}-P$_{tac}$ fragment of pJF118EH subcloned in [BamHI]-EcoRI-digested pME6031; *lacI*^{q}-P$_{tac}$ expression vector	16
pME6032-gacA	pME6032 derivate containing *gacA* gene	This study
pME6032-gacS	pME6032 derivate containing *gacS* gene	This study
pME6032-rsmX	pME6032 derivate containing *rsmX* gene	This study
pME6032-rsmY	pME6032 derivate containing *rsmY* gene	This study
pME6032-rsmZ	pME6032 derivate containing *rsmZ* gene	This study
Mutagenesis		
pME3087	ColE1-based suicide plasmid; Tc^r	41
pME3087-ΔgacA	pME3087 derivate containing upstream and downstream regions of *gacA*	This study
pME3087-ΔlapD	pME3087 derivate containing upstream and downstream regions of *lapD*	This study

interaction with plants. Three sRNAs (RsmX, RsmY, and RsmZ) were expressed under GacS-GacA control in *P. brassicacearum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. brassicacearum* strain NFM421 and its variants were grown at 30°C in tryptic soy broth (TSB) (Difco) or in 10-fold-diluted tryptic soy broth (TSB 1/10). For detection of extracellular protease activity, bacteria were plated on TSB 1/10 agar plates containing 1% skim milk. K10T-1 medium was prepared as previously described (28).

For growth on plates, media were solidified with 15 g/liter agar (Sigma). Pseudomonas agar F (PAF) (Difco) was used to reveal phenotypic switching. *Escherichia coli* strains GM2163, TOP10, and S17-1 were grown in Luria broth (LB) at 37°C.

Transcriptome analysis using homemade microarrays. Transcriptome analysis of the wild-type strain and variant V1 grown in TSB 1/10 medium during 24 h was performed using homemade DNA microarrays as previously described (31). Experiments were performed in triplicate.

DNA and RNA experiments. Chromosomal DNA from *P. brassicacearum* NFM421 was prepared by phenol-chloroform extraction. Plasmids were extracted with the QIAprep spin miniprep kit (Qiagen), and DNA fragments were purified from agarose gels with the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions. RNA was extracted using the RNAprotect bacteria reagent and RNeasy mini kit (Qiagen). Reverse transcription-PCR (RT-PCR) assays were done using the Transcriptor first strand cDNA synthesis kit (Roche).

5′ RACE. The 5′ ends of *rsmX* and *rsmY* transcripts were mapped by rapid amplification of cDNA ends (RACE). A reverse transcription reaction was performed using total RNA from *P. brassicacearum* strain NFM421 isolated in late stationary phase and the Transcriptor first strand cDNA synthesis kit (Roche). The 5′-phosphorylated, 3′-end cordecypin-blocked oligonucleotide DT88 (37) was ligated to the single-stranded cDNA with T4 RNA ligase (New England BioLabs). The anchor-ligated cDNA was amplified first with primers DT89 (anchor-specific primer) and an *rsm*-specific primer (see Table S1 in the supplemental material). Then, a nested PCR was carried out using DT89 and a second *rsm*-specific internal primer. Finally, the PCR product was cloned into the cloning vector pCR4Blunt-TOPO (Invitrogen). Three independent clones were sequenced using primer T7 (GATC Biotech).

Overexpression of sRNAs. To overexpress *rsmX*, *rsmY*, and *rsmZ*, we used the expression vector pME6032 (16), from which we removed the Shine-Dalgarno sequence, as this would have been unsuitable for the expression of noncoding sRNAs. The *rsmX*, *rsmY*, and *rsmZ* genes were amplified from NFM421 chromosomal DNA by PCR using the primers rsmX-PstI and rsmX-KpnI, rsmY-PstI and rsmY-KpnI, and rsmZ-PstI and rsmZ-KpnI, respectively (see Table S1 in the supplemental material), digested by PstI and KpnI, and inserted into PstI/KpnI-cut pME6032. The introduction of the PstI restriction site added 6 nucleotides (nt) of the RsmX sRNA at the 5′ end (5′-CTGCAGTCCACTGAA. . . instead of 5′-T

CCACTGAA. . .), added 4 nt and modified 1 nt of the RsmY sRNA at the 5′ end (5′-CTGCAGGGATGTAGCGC. . . instead of 5′-ATGGATGTAG CGC. . .), and added 6 nt to the RsmZ sRNA at the 5′ end (5′-CTGCAG TGTCGACGGA. . . instead of 5′-TGTCGACGGA. . .). All mutations were verified by sequencing.

Construction of chromosomal mutants. Upstream and downstream regions of *gacA* were amplified from *P. brassicacearum* NFM421 genomic DNA with the MD-gacA-1/MD-gacA-2 and MD-gacA-3/MD-gacA-4 primers, respectively (Table S1). In a second PCR, the overlap between primers MD-gacA-2 and MD-gacA-3 enabled amplification of a 2-kb fragment, in which the upstream and downstream regions were ligated together. This fragment was then cloned into the suicide vector pME3087 (41) and introduced into *E. coli* S17-1 by transformation. The recombinant plasmid was then introduced into the wild type by conjugation; cells were selected on TSB 1/10 agar plates supplemented with ampicillin (100 μg/ml) and tetracycline (100 μg/ml). Potential mutants were obtained by serial growth in TSB 1/10 broth and screening for tetracycline-sensitive cells. The resulting deletion in *gacA* was verified by PCR. The same technique was used for the construction of a *lapD* mutant with primers specified in Table S1 in the supplemental material.

Complementation of the *gacA* mutant and phase II variants. To express the *gacA* gene, we amplified it from chromosomal DNA by PCR using the primers gacA-EcoRI and gacA-XhoI (Table S1) and cloned it into plasmid pME6032. The resulting recombinant plasmid was used to complement the Δ*gacA* strain and phase II cells. Similarly, the *gacS* gene was inserted into pME6032 following amplification with the primers gacS-EcoRI and gacS-KpnI. The *gacA* and *gacS* genes were overexpressed by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Construction of transcriptional *lacZ* fusions. To construct transcriptional fusions, we amplified the promoter regions of *rsmX*, *rsmY*, and *rsmZ* by PCR with primers listed in Table S1 in the supplemental material. In these transcriptional fusions, the promoter fragments covered a region extending to 523 bp, 537 bp, and 279 bp upstream of the deduced transcription start site, respectively. The PCR products were then digested with appropriate restriction enzymes and cloned into pME6016 (35). The constructs were checked by sequencing.

β-Galactosidase assays. Cultures containing an *rsmX*-, *rsmY*-, or *rsmZ* promoter-*lacZ* construct were grown overnight, diluted 1:200 into 8 ml of TSB with tetracycline (20 μg/ml), and grown over a period of 24 h. For the time course of *rsmX*, *rsmY*, and *rsmZ* expression, 50-ml TSB cultures were sampled at different times and assayed immediately. β-Galactosidase activities were measured at different times by the Miller (27) method.

Metabolomics. Cells were harvested in late stationary phase and washed with ultrapure water. The lyophilized samples were sent for analysis to Munich and extracted in 50/50 methanol water in an ultrasonic bath for 15 min. The pellets were centrifuged at 14,000 rpm for 5 min, and the supernatant was analyzed on a Bruker-Daltonics APEXQ 12 T Fourier transform ion cyclotron resonance (ICR-FT) mass spectrometer (Bremen, Germany). The samples were introduced in infusion at a flow rate of 120 μl/h and ionized in negative and positive electrospray (ESI), and 512 scans were accumulated in broadband mass range (*m/z* 150 to 2,000). The instrument was externally calibrated on clusters of arginine on a daily basis, and the mass spectra were internally calibrated with fatty acids in negative ESI, allowing a maximum error of 250 ppb. Peaks exceeding a threshold signal-to-noise ratio of 3 were exported to peak lists and were submitted to a metabolite annotation Web interface, MassTRIX (www.masstrix.org). MassTRIX (36) processes the submitted mass peak list by comparing the input experimental masses against all compounds of the Kyoto Encyclopedia Genes and Genome (KEGG) chemical compound database using "*Pseudomonas putida F1*" as the model organism.

Biofilm assays. In a modification of the biofilm ring assay (30), overnight cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.05 in K10T-1 medium, and 1-ml samples were dispensed into glass tubes in triplicate. Following static incubation at 30°C, the medium was removed and tubes were washed gently with distilled water. Biofilm formation was visualized by using crystal violet staining.

TABLE 2 Spontaneous mutations found in *gacS* and *gacA* genes

Strain	Phase	Functional gene present		Remarks
		gacA	*gacS*	
NFM421 (wild type)	I	+	+	
Variants				
V1	II	+	−	2-bp deletion/frameshift ($AC_{1345\text{-}1346}/STOP_{467}$)
V2	II	−	+	Point mutation ($T_{11}\text{-}G_{11}/V_4\text{-}G_4$)
V3	II	+	−	Point mutation ($C_{894}\text{-}G_{894}/R_{298}\text{-}S_{298}$)
V4	II	−	+	Point mutation ($G_{112}\text{-}T_{112}/E_{38}\text{-}STOP_{38}$)

RESULTS

Phenotypic switching in *P. brassicacearum*. When the wild-type strain (= phase I cells) of *P. brassicacearum* is grown on rich medium for several days, phenotypic switching occurs, leading to the appearance of variants (= phase II cells). This phenomenon also occurs in the rhizosphere of *Arabidopsis thaliana* (2). To identify the regulatory components controlling phenotypic switching in strain NFM421, we initiated a transcriptomic analysis using homemade microarrays (31). The most remarkable changes differentiating the variants from the wild type were seen in transcripts related to biofilm formation as type IV pili and secondary metabolism as 2,4-diacetylphloroglucinol (DAPG) (see Table S2 in the supplemental material). In biocontrol strains of *P. fluorescens*, these traits are known to be positively regulated by the GacS-GacA system (15, 20, 23), which has been shown to be a frequent target of spontaneous mutations (9, 39). Therefore, we sequenced the *gacS* and *gacA* genes in the wild type (= phase I cells) and in four variants (Table 2); this analysis revealed that point mutations had occurred in *gacS* or *gacA* in all tested variants.

Complementation of two representative variants, V1 (*gacS* negative) and V2 (*gacA* negative), with the wild-type *gacS* and *gacA* genes, respectively (Table 2), restored the phase I phenotype, as evident from the restoration of protease activity, colony morphology, and biofilm formation (Fig. 1). Deletion of *gacA* in the wild type resulted in a phenotype that was indistinguishable from that of variants V1 and V2 (Fig. 1), and the phase I phenotype was fully restored upon introduction of the cloned $gacS^+$ gene in V1 or that of $gacA^+$ in V2. Thus, these results indicate that phenotypic switching in *P. brassicacearum* can be caused by mutations that occur in *gacS* or *gacA*.

Expression of the *rsmX*, *rsmY*, and *rsmZ* sRNA genes. Our microarrays contain DNA fragments from coding and noncoding regions, including *rsmY* and *rsmZ*. These genes were differentially expressed in the wild type and the variants (see Table S2 in the supplemental material). In addition, a BLAST search of the *P. brassicacearum* NFM421 genome against the *P. fluorescens* CHA0 *rsmX* gene with its flanking genes (encoding hypothetical proteins) revealed that in *P. brassicacearum* this genomic region is conserved. Transcriptional starts have been determined by 5′RACE for the *rsmX* (5′-TCCACTGAA. . .) and *rsmY* (5′-ATGG

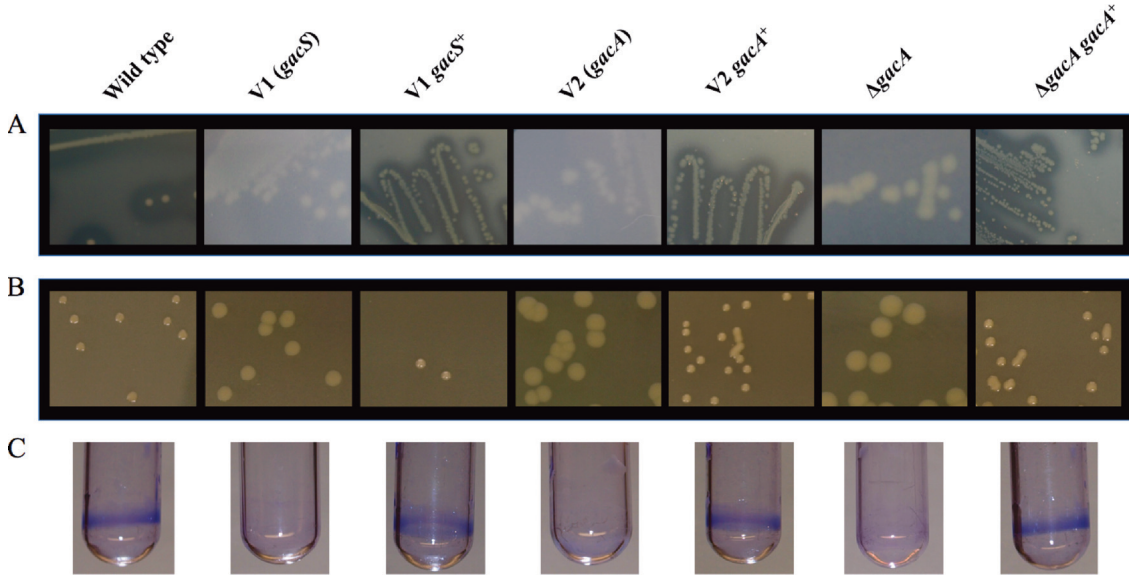

FIG 1 Restoration of the wild-type phenotypes by expression of *gacS*$^+$ (pME6032-*gacS*) in the variant V1 (*gacS*) or by expression of *gacA*$^+$ (pME6032-*gacA*) in the variant V2 (*gacA*) and in the Δ*gacA* mutant, as illustrated by protease activity on skim milk medium (A), colony morphology on PAF medium (B), or biofilm formation (C).

ATGTAG. . .) genes. The transcriptional start of *rsmZ* was identical to that from *P. fluorescens* CHA0 (5′-TGTCGACGGA. . .) (16). Expression analysis of *rsmX*, *rsmY*, and *rsmZ* using RT-PCR shows that these three genes are transcribed in growing wild-type cells but not in the *gacS*-impaired variant V1 (Fig. 2) nor in the *gacA*-impaired variant V2 (data not shown). We then determined *rsmX*, *rsmY*, and *rsmZ* expression during growth in TSB medium by measuring β-galactosidase activities of transcriptional *rsmX-lacZ*, *rsmY-lacZ*, and *rsmZ-lacZ* fusions in the wild type and in the variant V1 (Fig. 3). In the late exponential phase, the expression of the *rsmX-lacZ* and *rsmZ-lacZ* fusions increased sharply compared with that of the *rsmY-lacZ* fusion. These data were confirmed with the Δ*gacA* mutant (Table 3). Altogether, these results indicate that unlike *rsmZ* and *rsmY*, the expression of *rsmX* is completely controlled by the GacS-GacA system.

In *P. fluorescens* CHA0, the sRNAs RsmX, RsmY, and RsmZ act as antagonists of two similar RNA-binding proteins, RsmA and RsmE (20, 33). Homologues of the *rsmA* and *rsmE* genes are present in the *P. brassicacearum* NFM421 genome; the corresponding proteins share more than 95% identity with their *P. fluorescens* counterparts (see Fig. S1 in the supplemental material). By analogy with the *P. fluorescens* model, it is possible that the main function of RsmX, RsmY, and RsmZ is to relieve repression of target mRNA translation by RsmA and RsmE.

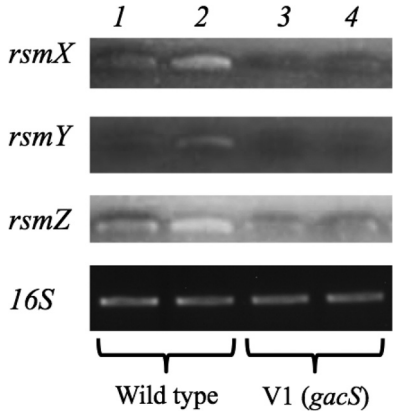

FIG 2 Expression of *rsmX*, *rsmY*, and *rsmZ* in the wild type and in the variant V1 (*gacS*) determined by RT-PCR during exponential phase (4-h growth culture) (lanes 1 and 3) or stationary phase (24-h growth culture) (lanes 2 and 4).

Overexpression of *rsmX*, *rsmY*, or *rsmZ* suppresses the phenotypic switch. When either *rsmX*, *rsmY*, or *rsmZ* was overexpressed from a plasmid under the control of an IPTG-inducible exogenous promoter, the GacS-GacA system defect in the variant V1 was suppressed, as revealed by restoration of protease activity, colony morphology, and biofilm formation (Fig. 4). The same results were obtained with the variant V2 (data not shown), indicating that in *P. brassicacearum* the three sRNAs are functionally redundant with respect to their ability to relieve the effects of a *gacS-gacA* mutation.

Toward a description of the GacS-GacA regulon in *P. brassicacearum*. Previous studies have shown that phenotypic switching from phase I to phase II in *P. brassicacearum* involves downregulated transcription of *lipA* (lipase), *phlACBD* (DAPG biosynthesis), and *hcnABC* (hydrogen cyanide biosynthesis) (12, 31). Using *lipA*, *phlD*, and *hcnB* as molecular markers, we confirmed by RT-PCR that the transcriptional expression of these genes was low in the *gacS*-negative variant V1 by comparison with the wild type (Fig. 5). Among the genes that are downregulated in phase II, some are involved in adherence and biofilm formation, such as *pilA* and *pilR*, which participate in the synthesis of type IV pili in *P. aeruginosa* (26). In *P. brassicacearum*, the expression of these genes was much higher in phase I than in phase II (Fig. 5), which is consistent with the better biofilm formation ability of phase I cells (Fig. 1). In addition, we noticed differential expression of *algD* (encoding GDP-mannose dehydrogenase, the key enzyme of the alginate biosynthesis [32]) (Fig. 5). This result

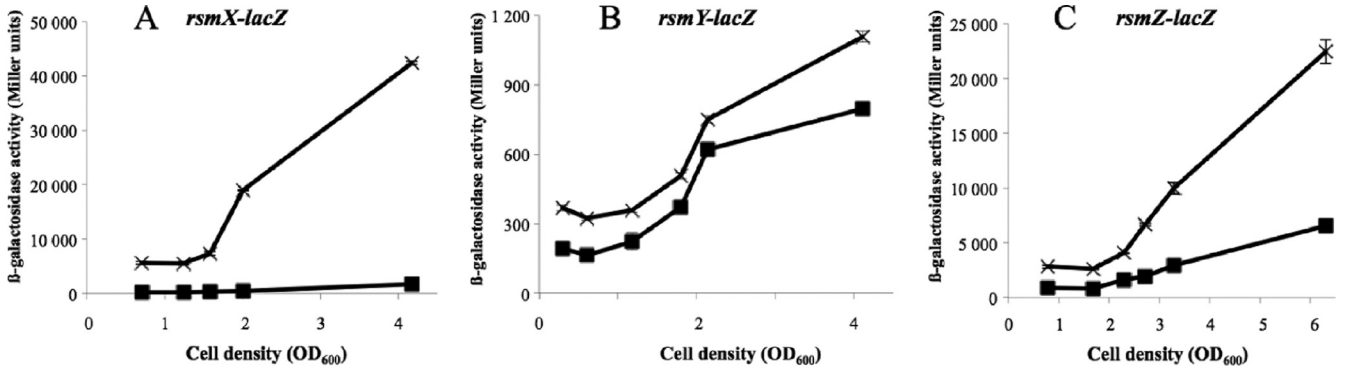

FIG 3 Growth-phase-dependent expression of *rsmX*, *rsmY*, and *rsmZ*. β-Galactosidase measurements were performed on a transcriptional *rsmX-lacZ* fusion (A), a transcriptional *rsmY-lacZ* fusion (B), and a transcriptional *rsmZ-lacZ* fusion in the wild type (×) and in the variant V1 (*gacS*) (■) (C). Each result is the mean ± standard deviation of three measurements.

was confirmed with the MassTRIX annotation, where GDP-mannuronate was observed in phase I cells but not in phase II cells. Thus, the GacS-GacA system appears to act as an activator of alginate production in *P. brassicacearum*, similar to the situation in *Azotobacter vinelandii*, a close relative of *P. fluorescens*, where alginate production is positively regulated by GacA and RpoS (11).

In *P. fluorescens* WCS365, the LapD inner-membrane protein is required for adherence and biofilm formation via maintenance of the LapA adhesin on the cell surface (17). In *P. brassicacearum*, a *lapD* mutant was impaired in biofilm formation (data not shown). However, in this mutant the *rsmX*, *rsmY*, and *rsmZ* genes were normally expressed (Table 4). Thus, while both LapD and the GacS-GacA system are required for biofilm formation, these regulators apparently do not belong to the same signal transduction pathway.

Three gene clusters encoding type VI secretion machineries and termed Hcp secretion islands (HSIs) have been identified in *P. aeruginosa* (10). In *P. brassicacearum*, two of these clusters (HSI-I and HSI-III) are well conserved. The expression of the *hcp1* and *hcp3* genes, encoding potentially secreted Hcp proteins, was downregulated in the *gacS*-negative variant V1, by comparison with the wild type (Fig. 5). In addition, the RT-PCR data show that these *hcp* genes were differently expressed during growth, in that *hcp1* was highly transcribed in the exponential phase, whereas *hcp3* was mainly expressed in the stationary phase. A third *hcp* gene (*hcp2*) of *P. brassicacearum*, which is similar to *hcp2* of *P. aeruginosa*, was not under GacS-GacA control (Fig. 5). Furthermore, transcriptomic data revealed that a gene cluster involved in chemotaxis was downregulated in variant V1 (see Table S2 in the supplemental material). Expression analysis by RT-PCR confirmed that at least two genes of this cluster, *cheA* (for a histidine kinase) and PSEBR_a3442 (for a methyl-accepting chemotaxis protein MCP), were under positive control by the GacS-GacA system (Fig. 5).

Metabolite analysis showed that the synthesis of DAPG, indoleacetic acid (auxin), and three different *N*-acyl-homoserine lactones (AHLs) (namely, hydroxydecanoyl-homoserine lactone, tetradecanoyl-homoserine lactone, and hydroxytetradecanoyl homoserine lactone) and GDP-D-mannuronate, "a precursor of alginate," was under positive control of GacA in *P. brassicacearum* (Fig. 6). Auxin production by strain NFM421 has previously been observed to modify the root architecture of *A. thaliana* (3), whereas the AHLs may be part of a quorum sensing system. However, the genes required for the synthesis of these metabolites have not yet been identified. In conclusion, the expression data obtained here point to a wide-ranging impact of the GacS-GacA system in *P. brassicacearum*.

TABLE 3 Influence of *gacA* deletion on *rsm* expression[a]

Gene	Miller units of β-galactosidase activity		
	Phase I	V1 (*gacS*)	Δ*gacA*
rsmX	42,440 ± 1,623	1,765 ± 148	1,231 ± 88
rsmY	1,430 ± 199	947 ± 98	1,332 ± 71
rsmZ	22,461 ± 1,076	6,573 ± 132	8,016 ± 72

[a] Activities of a *lacZ* reporter fused to promoter regions of *rsmX*, *rsmY*, and *rsmZ* were measured in *P. brassicacearum* wild-type (Phase I), *gacA* mutant (Δ*gacA*), and variant V1 (*gacS*) strains and are shown as Miller units of β-galactosidase activity (three measurements, means ± standard deviations). Cultures were grown in 50 ml of TSB medium for 24 h at 30°C before the assay.

DISCUSSION

We have discovered that in *P. brassicacearum* the GacS-GacA two-component system controls phenotypic switching, which contributes to bacterial adaptation to the rhizosphere and allows the bacterial population to explore the whole root system and to exploit the root resources (2). During root colonization at primary stages when root exudates are available, wild-type (phase I) cells take advantage of forming protecting biofilms and producing exoproducts. When the roots grow, exudates are less abundant and nutrients are rapidly depleted. Phenotypic switching through mutation in *gacA* or *gacS* enables the cells to become more motile and to colonize secondary roots and root tips more effectively (2). Similarly, in *P. fluorescens* F113, the GacS-GacA two-component system positively regulates biofilm formation on inert material (but not on plant roots) and negatively regulates flagellar motility (4).

Our findings show that single spontaneous mutations in the *gacS-gacA* system lead to drastic pleiotropic changes. In particular, the expression of secondary metabolites (e.g., the antifungal compounds DAPG and cyanide), auxin, exoenzymes (e.g., lipase and protease), AHLs, the type VI secretion machinery, and alginate was downregulated, and biofilm formation ability was greatly reduced. Mutations in the *gacS-gacA* system have been previously reported to occur frequently in other *Pseudomonas* species. These

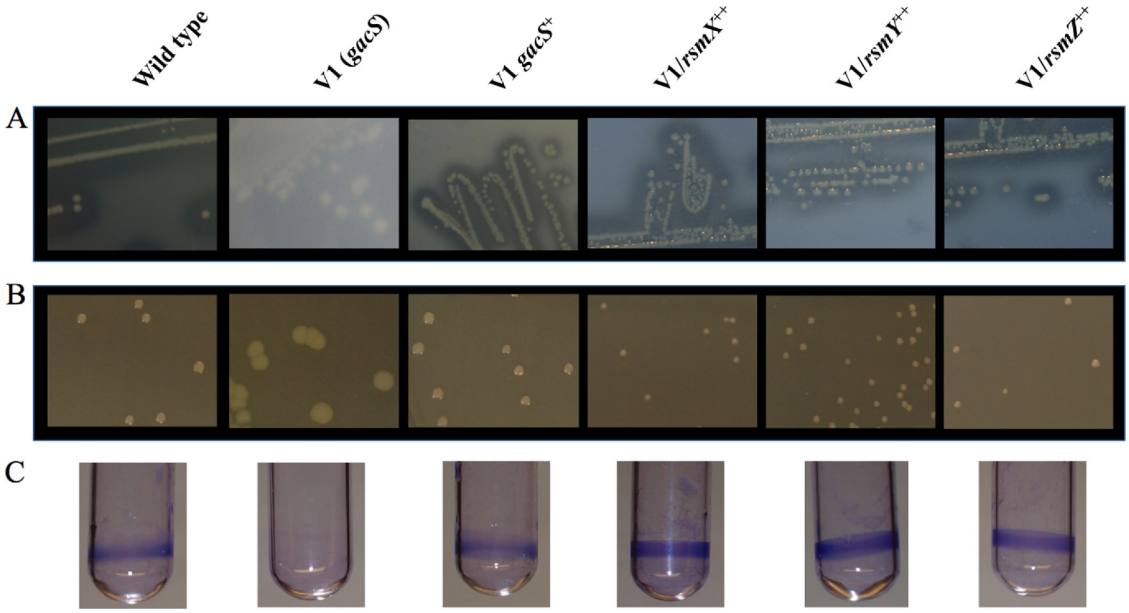

FIG 4 Restoration of the wild-type phenotypes by overexpression of either *rsmX* (pME6032-*rsmX*), *rsmY* (pME6032-*rsmY*), or *rsmZ* (pME6032-*rsmZ*) in the variant V1 (*gacS*), as determined by protease activity on skim milk medium (A), colony morphology on PAF medium (B), or biofilm formation (C).

mutations occur spontaneously and include point mutations, small insertions and deletions, and large rearrangements in *gacS* or *gacA* (9, 39).

In *Pseudomonas* species, the GacS-GacA system controls the synthesis of several sRNAs and thereby influences the expression of several hundred genes (7, 15, 23). In *P. brassicacearum*, we found evidence for three such sRNAs: RsmX, RsmY, and RsmZ. Measurements of β-galactosidase activities of transcriptional *rsmX-lacZ*, *rsmY-lacZ*, and *rsmZ-lacZ* fusions (Fig. 3) in strain NFM421 showed differential expression patterns, suggesting that in addition to the GacS-GacA system further regulators may be involved in regulating transcription of *rsmX*, *rsmY*, and *rsmZ*.

In *P. aeruginosa* and *P. fluorescens* CHA0, two sensor kinases, LadS and RetS, inversely influence the output of the GacS sensor (18, 40). Two other regulators, PsrA and HptB, have recently been shown to modulate *rsmZ* and *rsmY* expression, respectively. PsrA, an activator of the stress and stationary-phase sigma factor RpoS, also directly activates *rsmZ* transcription in *P. fluorescens* (19), whereas HptB indirectly controls *rsmY* expression in *P. aeruginosa* (6). Homologues of these four regulator genes are present in the *P. brassicacearum* genome and may participate in fine-tuning the GacS-GacA regulatory pathway.

In several *Pseudomonas* species, the GacS-GacA two-component system positively controls the quorum-sensing machinery via *N*-acyl-homoserine lactones (21, 23). In this study, we identified three different AHLs in *P. brassicacearum*. They were produced in

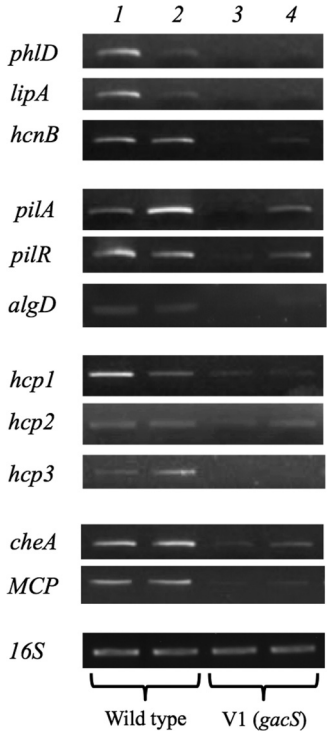

FIG 5 Expression of genes involved in secondary metabolism, biofilm formation, type VI secretion, and chemotaxis in the wild type and in the variant V1 during exponential phase (lanes 1 and 3) and stationary phase (lanes 2 and 4), as determined by RT-PCR.

TABLE 4 Influence of LapD on *rsm* RNA expression[a]

Gene	Miller units of β-galactosidase activity	
	Phase I	Δ*lapD*
rsmX	22,111 ± 2,990	22,735 ± 39
rsmY	1,454 ± 90	1,339 ± 11
rsmZ	5,390 ± 623	5,478 ± 152

[a] Activities of a *lacZ* reporter fused to promoter regions of *rsmX*, *rsmY*, and *rsmZ* were measured in *P. brassicacearum* wild-type (Phase I) and *lapD* mutant (Δ*lapD*) strains and are shown as Miller units of β-galactosidase activity (three measurements, means ± standard deviations). Cultures were grown in 8 ml of TSB medium for 24 h at 30°C before the assay.

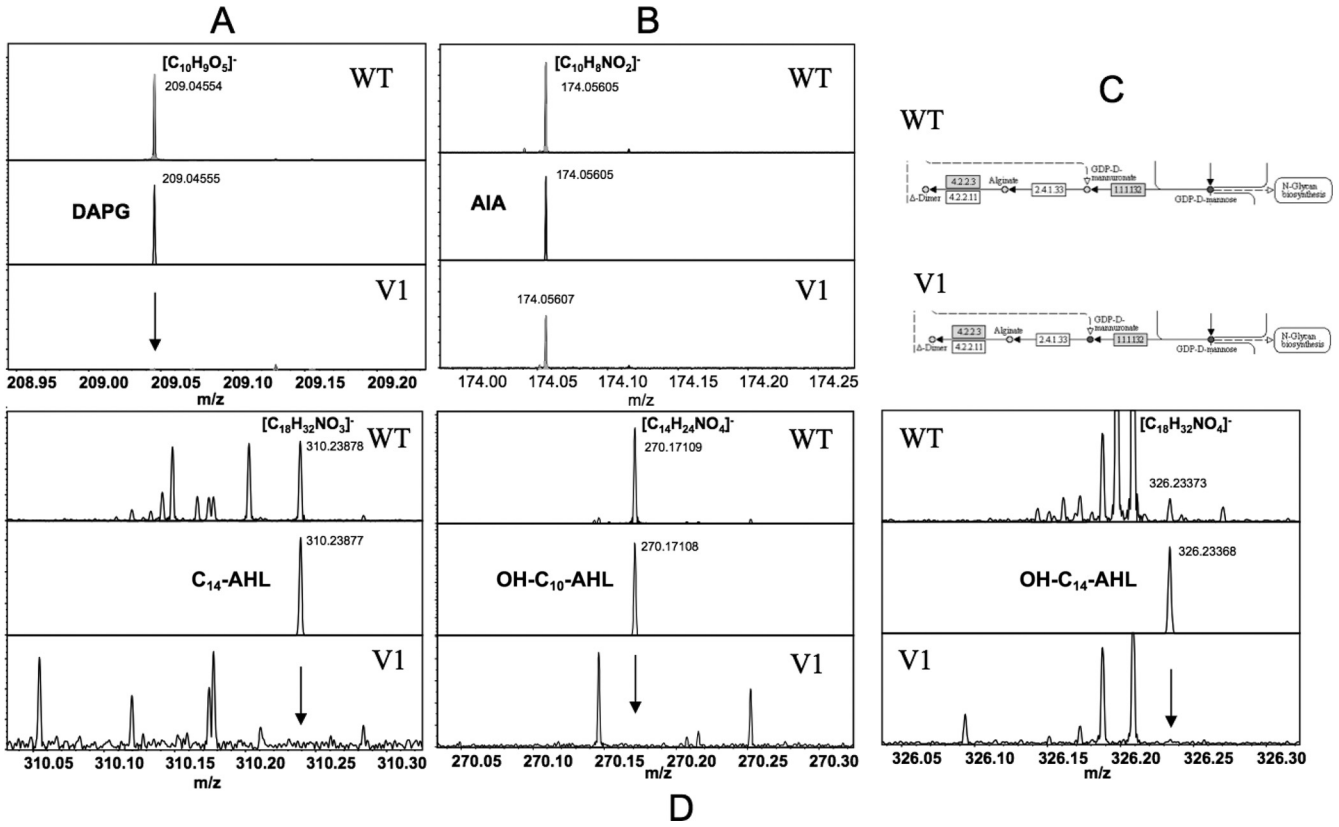

FIG 6 ICR-FT/mass spectrometry (MS) analysis for the wild type (WT) or variant V1 *gacS* (V1) of DAPG (A), indole-3-acetic acid (AIA) (B), GDP-D[SCAP]-mannuronate (C), a precursor of alginate from the fructose and mannose metabolism pathway (enzyme 1.1.1.132, corresponding to AlgD) that is detected only with wt strains (a filled circle indicates the production of the metabolite, whereas an open circle indicates that it was not detected), or C_{14}-AHL, OH-C_{10}-AHL, and OH-C_{14}-AHL (D).

significantly larger amounts by the wild type than by the variants, indicating positive control by GacA of the quorum sensing system in this organism.

The precise functions of the type VI secretion system are poorly understood at present. It appears to be involved in many processes, including virulence, biofilm formation, and stress response. In *P. aeruginosa*, an important function of this secretion system is to provide a competitive advantage over other bacteria. In the same organism, GacA regulates expression of *hcp1* positively and that of *hcp3* negatively, whereas that of *hcp2* is GacA independent (5). A different picture is observed in *P. brassicacearum*, where GacA positively regulated both *hcp1* and *hcp3*. Additional fine-tuning regulation is suggested for *hcp1*, which was mainly transcribed during the exponential phase, whereas *hcp3* transcription increased over time to reach a maximum at the end of growth. In contrast, *hcp2* expression was GacA independent in *P. brassicacearum*. Altogether, these data support the hypothesis that the different Hcp secretion islands are not redundant and may fulfill different functions.

In conclusion, the fact that overexpression of either *rsmX*, *rsmY*, or *rsmZ* suppressed a *gacA* defect (Fig. 4) indicates that these three sRNAs represent a major positive control elements in the GacS-GacA cascade of *P. brassicacearum* NFM421.

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